Predisposition of Melaleuca (Melaleuca quinquenervia) to Invasion by the Potential Biological Control Agent Botryosphaeria ribis¹

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Abstract. Enhancement of the canker causing ability of Botryosphaeria ribis on melaleuca was studied with respect to stress from simulated drought, low temperature, and defoliation treatments. Low xylem water potential was related to increased level of canker development and subsequent tree mortality. Canker development was enhanced by low temperature treatments with alternating exposure to 6 C for 3 d followed by 4 d at 30 (\pm 5) C for 8 wk. Partial defoliation did not affect canker development but complete defoliation of B. ribis-inoculated ramets resulted in tree mortality within 4 wk. Callusing of melaleuca wounds was either reduced or prevented in stressed trees. These observations suggest that stress induced on the tree enhances the tree-killing efficacy of this fungus. Nomenclature: Melaleuca, Melaleuca quinquenervia (Cav.) Blake #3 MLAQU.

Additional index words: Defoliation, drought, low temperature, stem canker, MLAQU.

INTRODUCTION

Melaleuca (other common names: paperbark tree, cajeput tree) is an invasive weed tree in the Everglades and surrounding areas in South Florida (4, 5). It is a prolific seed producer, aggressively colonizing disturbed and undisturbed sites, displacing native vegetation, degrading wildlife habitat, and causing a number of human health problems (10, 12). Current containment and eradication strategies utilize relatively expensive mechanical and chemical measures (4, 5). Long-term impacts of chemical measures in the Everglades are unknown (18). Development of an environmentally sensitive biological control strategy is receiving increased attention, and the suitability of melaleuca as a target weed for biological control has been discussed (4). Several herbivorous insects are under evaluation as potential agents of biological control⁴.

Few fungal pathogens have been recorded on melaleuca (1). To date, only the *Fusicoccum* anamorph of *Botryosphaeria ribis* Gross & Duggar, has been tested for pathogenicity on this tree

(18). The suitability of an indigenous fungus as a mycoherbicidal candidate for biological control of weeds in respect to environmental safety has been discussed by Charudattan (6). Botryosphaeria ribis is recognized as a pathogen on many tree species (19,24). Despite being indigenous to the area and pathogenic to melaleuca (18), this fungus is not known to cause large-scale epiphytotics on field populations of this tree species. It is hypothesized that the susceptibility of melaleuca to B. ribis may be strongly influenced by the occurrence of one or more stress factors that favor pathogen invasion by reducing host resistance. Identification of such factors may be important to the development of an integrated approach with herbivorous insects and pathogenic fungi to control melaleuca.

The role of stress factors in predisposing woody plants to various pathogens is well established. Water stress of various magnitudes (2, 3, 7, 9, 16) and ranges of low temperature (11, 16, 23, 27) are well-known stresses that enhance disease progress in various host-pathogen combinations. Similarly, repeated defoliation during the growing season has been reported to weaken and predispose trees to invasion by various pathogenic organisms (7, 16, 21, 23, 26, 27), and subsequently to favor canker development and tree mortality.

The objectives of this research were to evaluate the effects of drought, low temperature, and defoliation treatments on the occurrence and severity of canker disease caused by *B. ribis* on melaleuca.

MATERIALS AND METHODS

Isolate acquisition. During 1989-90, six isolates of *B. ribis* (BR-1 through BR-6) were obtained from declining melaleuca trees on the Acme-2 management unit of the Loxahatchee National Wildlife Refuge in Palm Beach County, Florida. These isolates were single-spored (macroconidia) and were tested for pathogenicity on clones of melaleuca; isolates BR-2 and BR-5 were found to be the most and least virulent, respectively (18). These two isolates were used in this research.

Tree clone production. Stem cuttings were obtained from two randomly selected melaleuca trees located at least 50 m apart in the Acme-2 management unit. The cuttings were rooted by placing them in water in buckets and then transferred to 5-gal plastic containers containing a mixture of peatmoss and sand (1:1). These clones were designated as MQ-1 and MQ-2. Cuttings from these ramets were rooted to produce additional ramets which were transferred to 1-gallon plastic containers containing peatmoss and sand (1:1). These additional ramets grew for 18 mo and were used in inoculation experiments.

Inocula preparation and inoculation techniques. Cultures of BR-2 and BR-5 were grown on potato-dextrose broth⁵ with

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³Letters following this symbol are a WSSA-approved computer code from Composite List of Weeds, Revised 1989. Available from WSSA, 1508 West University Ave., Champaign, IL 61821-3133.

⁴T.D. Center, USDA-ARS, Fort Lauderdale, FL. Unpublished data.

⁵Difco, Detroit, MI 48232, USA.

continuous shaking (100 rpm) for 5 d, filtered through cheese-cloth, washed with deionized water⁶, blended for 25 s using a commercial blender, and adjusted to 1% dry wt/volume by adding DW (18). These steps were performed under sterile conditions. Melaleuca ramets were inoculated at the mid-height of the main stem by creating a small wound (1.5-mm diam and 2-mm deep drill-hole beneath the bark surface) and filling it with 1% hyphal suspension. The wounds were wrapped with Parafilm⁷ immediately after inoculation. Ramets were randomized by location and maintained at 30 (±5) C on a greenhouse bench unless otherwise noted.

Simulated drought treatments. Tree clone MQ-1 and the two isolates of *B. ribis* were used to evaluate the effects of drought on canker development. Four treatments were used to create different levels of xylem water potential. These four treatments were composed of ramets watered daily (Trt.1), every 3 d (Trt.2), every 7 d (Trt.3), and every 12 d (Trt.4) to field capacity for 8 wk. Five ramets were used for each isolate in each treatment group. The trees were maintained in the greenhouse for the 8-wk experimental period.

Ramets in each treatment were exposed to their respective watering regime one time prior to inoculation. After inoculating, the appropriate watering regime was followed throughout the 8-wk experimental period. Xylem water potential of one twig from approximate mid-height of the saplings for each of three plants per treatment was measured using a pressure bomb⁸ at 7:30 to 8:30 AM before and 24 h after watering to field capacity. During measurement of xylem water potential, the ambient temperature in the greenhouse was ca 25 C. Ramets were evaluated every week for permanent wilting symptoms (not reviving within 24 h after watering to field capacity). The number of wilted ramets were counted, harvested, and the canker length was measured after splitting stems longitudinally through the point of inoculation. After 8 wk, the remaining ramets were harvested, the stems split through the point of inoculation, and the proximal and distal canker lengths were measured.

Low temperature treatments. The effect of low temperature treatments on disease development was determined by subjecting clone MQ-2 to four treatments for 8 wk: exposed to 30 (\pm) C continuously (Trt.1), 6 C for 3 continuous d wk⁻¹ (Trt.2), 6 C for 6 continuous d wk⁻¹ (Trt.3), and 0 (\pm 1) C for 16 h wk⁻¹ (Trt.4). Each treatment for isolates BR-2 and BR-5 was composed of five ramets. Ramets in each treatment were exposed once to their respective low temperature treatments prior to inoculation with B. ribis. After inoculations, treatments 2, 3, and 4 (assigned to low temperature treatments) were maintained together on the greenhouse bench at 30 (\pm 5) C for the remainder of a week. When the ramets were on the greenhouse bench, they were watered daily to field capacity and evaluated for wilt symptoms. Methods for canker measurement were the same as in the "Simulated drought treatments" experiment.

Defoliation treatments. Effects of defoliation treatments on canker development were determined using tree clone MQ-2 and isolates BR-2 and BR-5 of *B. ribis*. Treatments were 0 (Trt.1), 50 (Trt.2), and 100 (Trt.3) percent defoliation of the total leaf area. Each treatment for each isolate was represented by five ramets. Additionally, five check plants per isolate were maintained for comparison with Trt.3. These five check plants were completely defoliated and wounded by drilling a 1.5-mm diam and 2-mm deep drill-hole beneath the bark surface but they were not inoculated with the fungus.

Ramets in treatments 2 and 3 were defoliated by removing the appropriate proportion of the area of new leaves every week for 2 wk. After 2 wk, ramets were inoculated at the mid-height of the main stem using the same inocula and inoculation techniques described for "Simulated drought treatments" experiment. The ramets in each treatment were maintained separately on a greenhouse bench at 30 (±5) C and daily watering schedule. Thereafter, the level of defoliation among treatments was maintained by removing an appropriate area of new leaves every week for 4 wk. Ramets in all three treatments were harvested 4 wk after inoculation because the majority of ramets in treatment 3 were dead. Canker lengths were measured in treatments 1 and 2. However, only percentage mortality was recorded for the checks and the fungus-inoculated ramets in treatment 3. Canker length could not be recorded due to advanced tissue deterioration.

Statistical analysis. Total canker length (proximal + distal) constituted the response variable for all experiments. Analysis of variance and mean separations among variables were performed using GLM procedures in SAS (20). Host clones (ramets) and inocula (isolates) represented fixed effects in all experiments. Treatments were replicated five times within each experiment.

RESULTS AND DISCUSSION

Simulated drought treatments. The xylem water potential in the stem reflects the free water content in the xylem. A more negative water potential in the stem favors increased tissue colonization by the pathogens (23) due to reduced chemical and structural defense against them (17). Xylem water potentials of ramets watered every 7 d (Trt.3) or 12 d (Trt.4) became more negative both before and after watering, more so than ramets watered daily (Trt.1) or every 3 d (Trt.2) (Figure 1).

These differences in xylem water potential were related to canker length. Isolate and simulated drought treatment affected canker length, but the two main effects did not interact (Table 1). In drought-stressed trees, isolate BR-2 appeared more aggressive than isolate BR-5 (Table 2). Canker length increased significantly among treatments when xylem water potential reached a maximum of -1.5 MPa before or -0.6 MPa after watering (Figures 1 and 2). Similarly, an increase of canker dimensions with increased moisture stress (23) has been reported for B. dothidea (Moug ex. Fr.) Ces. et de Not. on European white birch (Betula alba L.) (7) and for Hypoxylon pruinatum (Klotz.) Cke. on Populus tremuloides Michx. (3).

After reaching a threshold of about -1.7 MPa, further decreases of xylem water potential did not affect canker length

⁶Abbreviations: DW, deionized water; NIT, nonsuberized impervious tissue.

⁷American National CanTM, Greenwich, CT 06830, USA.

⁸Plant Moisture Stress Inc., Corvallis, OR 97331, USA.

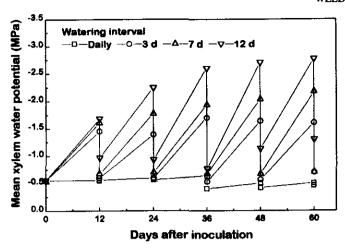


Figure 1. Xylem water potential of differentially moisture stressed stems of melaleuca ramets inoculated with B. ribis. Upper and lower points for each treatment in each observation represent xylem water potentials immediately before and 24 h following watering to field capacity.

(Figures 1 and 2). However, as xylem water potential reached -1.8 MPa before watering, the ramets in treatments 3 and 4 began to shed older leaves 4 wk after inoculation (Figure 3A), and 50 percent of the plants in these treatments were wilted. A similar phenomenon of shedding older leaves was observed among European white birch seedlings inoculated with B. dothidea (7).

Susceptibility of poplars to Cytospora chrysosperma (Pers.) Fr. increases when callusing of wounds is inhibited by low moisture stresses (17). In our study, callus ridges were prominent among ramets of treatments 1 and 2 (Figure 3B), and no mortality was observed. In treatments 3 and 4 (Figure 3C), callus ridges were initiated within a week but further development did not occur. Plants showing wilt symptoms developed extensive necrosis of the cambium and phloem. Removal of bark from

Table 1. Analysis of variance of data of experiments designed to assess the effects of stress factors on canker length on melaleuca stems at 8 wk after inoculation with isolates BR-2 and BR-5 of B. ribis.

Experiments	Sources	DF	F	Pr > Fa
Simulated	Isolate	1	14.79	0.0002
drought	Treatment	3	9.08	0.0001
	Isolate + treatment	3	0.89	0.1216
	Error	32		
	lsolate	1	10.32	0.0030
Low	Treatment	3	3.58	0.0244
temperature	Isolate + treatment	3	0.55	0.6491
	Error	32		
Defoliation	Isolate	1	15.61	0.0011
	Treatment	1	0.73	0.4060
	Isolate + treatment	1	1.22	0.2850
	Error	16		

 $[^]a$ Significance was determined at P = 0.05, and n = 5. Under "Defoliation," only treatments 1, (0% defoliation) and treatment 2 (50% defoliation) were used in this analysis.

Table 2. Main effect means and mean separations from analyses of variance for effects of stress factors on canker length (mm) on the stems of melaleuca inoculated with isolates of B. ribis (BR).

Experiments/ treatments	Fungal isolates	Mean canker length ^a
Simulated drought	BR-2	68.60 a
, and the second	BR-5	45.70 b
Low temperature	BR-2	72.42 a
	BR-5	43.90 b
Defoliation	BR-2	60.67 a
	BR5	52.33 b

^aMeans within an experiment followed by the same letter are not different according to Scheffe's test at P = 0.05. For each isolate in simulated drought and low temperature treatment experiments, n = 20; and for defoliation treatment experiments, n = 15.

noncallused stems revealed black necrotic phloem, cambium, and sapwood beyond the points of inoculations (Figure 3D).

Simulated drought stress promoted infection, colonization, and canker development in trees inoculated with *B. ribis*. Ramets of melaleuca that were watered daily expressed some resistance to fungal growth through xylem and phloem tissues. Our observation supports the finding that hyphae of *B. dothidea* in drought-stressed European white birch had extensive growth in the xylem vessels, in contrast to restricted growth in cells of similar tissues of nonstressed plants (14).

Simulated drought stress may have reduced the ability of the host to produce pathogen-inhibiting chemicals within ramets exposed to watering intervals of 7 (Trt.3) and 12 d (Trt.4), and subsequently allowed enhanced fungal growth in the stem tissues as indicated by canker lengths. Stress factors that cause low carbohydrate levels in the stem tissues have been reported to inhibit compartmentalization responses and callus formation and allow active invasion of the stem tissues (16). Puritch and Mul-

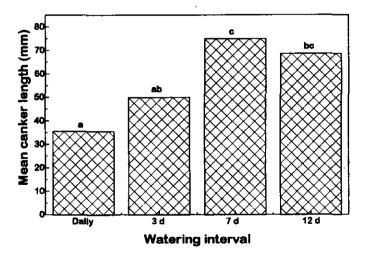


Figure 2. Effects of low moisture on canker development on the stems of metaleuca ramets incobulated with B. ribis. Bars with the same letter(s) are not different at P = 0.05 (Scheffe's test).



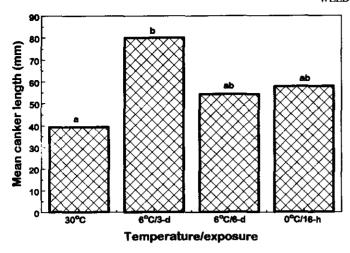
Figure 3. Effects of simulated drought treatments on canker disease in melaleuca caused by *B. ribis*. **A,** ramets in four treatments, inoculated with *B. ribis* and watered daily (Trt.1), every 3 d (Trt.2), every 7 d (Trt.3), and every 12 d (Trt.4); note progressive crown thinning (from Trt.1 to Trt.4) due to leaf-drop. **B,** a stem showing a typical canker among ramets wounded and inoculated with B. ribis and watered daily; note callus ridge (arrow) surrounding a canker. **C,** a stem showing a typical canker among ramets wounded and inoculated with B. ribis and watered every 12 d. Note lack of callus and the presence of blackened tissues (arrow). **D,** inner tissues in a typical stem canker among ramets wounded and inoculated with B. ribis and watered every 12 d; note blackened bark (arrow) and sapwood (arrow head).

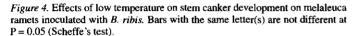
lick (17) found that the formation of NIT is a nonspecific reaction to wounding whose formation is delayed among drought-stressed plants. Lack of NIT around a wound facilitates the invasion of healthy tissues by pathogenic organisms (17). Similar host-pathogen interactions may have occurred in the *B. ribis*-melaleuca relationship.

Low temperature treatments. Considerable literature exists on the effect of low temperature treatments on disease development among woody plants. Almost all of those studies relate to the effects of freezing temperature on the creation of wounds, i.e., as infection courts for the establishment of canker incitants (11, 22). In our study, plants were exposed to above freezing temperatures (6 C) for different periods of time, or to 0 C for 16-h, after which they were incubated at 30 (\pm 5) C. These treatments were selected to reflect temperature regimes that may be encountered in field conditions.

Isolates and low temperature treatments affected canker length, but the interaction between isolates and low temperature

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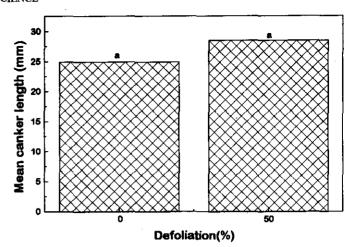


Figure 5. Effects of defoliation on canker development on melaleuca stems inoculated with B. ribis. Bars with the same letter(s) are not different at P = 0.05 (Scheffe's test).

treatments was not significant (Table 1). Overall, isolate BR-2 induced more tissue necrosis than did isolate BR-5 (Table 2). Canker development was greater among stressed ramets in treatment 2 (exposed 3 d wk⁻¹ to 6 C) than in nonstressed ramets in treatment 1 (Figure 4). Stressed ramets in treatments 3 and 4 developed smaller cankers than the stressed ramets in treatment 2. Also, sapwood discoloration was extensive in treatment 2.

Wound-callus was rudimentary among most stressed ramets of treatments 2, 3, and 4 (as shown in Figure 3C) compared to the nonstressed ramets in treatment 1 (as shown in Figure 3B), where cankers were surrounded by pronounced ridges of callus.

Continuous exposure of ramets for 16 h to 0 C caused stem freezing but resulted in neither visible cracks nor ramet mortality. Discoloration of sapwood was greater among ramets in treatment 2 which received alternating 3 d exposures to 6 C in the cold room and about 30 C in the greenhouse than the ramets in other treatments (Figure 4). Alternate low and high temperatures may have favored hyphal growth during the warm period, while exposure to low temperature induced a long-term inhibitory effect on host resistance. The 6 d wk⁻¹ exposure to 6 C (treatment 3) resulted in reduced canker development, presumably through negative effects on both host and pathogen. Wene and Schoeneweiss (27), reported an increased susceptibility of the frozen portion of stems of 2-year-old European mountain ash (Sorbus aucuparia L.) trees which were differentially exposed to low temperature treatments (up to -30 C) and inoculated with B. dothidea.

Ramets maintained continuously for 56 d in the greenhouse at 30 (\pm 5) C produced callus which appeared to slow the invasion potential of the fungus. Though callus formation was initiated among ramets exposed to 6 C for 3 d wk⁻¹, 6 C for 6 d wk⁻¹, and 0 (\pm 1) C for 16 h wk⁻¹ for a total of 8 wk, further development was arrested. Visually detectable wound-callusing occurred on 100, 10, 0, and 50% of the melaleuca ramets in treatments 1, 2, 3, and 4, respectively. In general, the low temperature treatments

appeared to inhibit callus formation and thus reduce host resistance to *B. ribis*. Wound-periderm formation in almond (*Prunus dulcis* (Mill.) Webb) trees (8) and wound response in the bark of three conifer species (15) is much slower at low than at high temperatures.

Defoliation treatments. Defoliation has been shown to predispose plants to invasion by various pathogenic organisms (16, 21, 23, 26). Schoeneweiss (23) stated that defoliation at a critical period or repeated defoliation over several years may result in predisposition of plants to otherwise nonaggressive pathogens. In our study, the magnitude of canker development at 0 and 50% defoliation was not different (Figure 5). In these two defoliation treatments, no ramet mortality occurred during the 4-wk experimental period. Ramets not defoliated (0%) or defoliated 50% developed a substantial callus around the inoculated wound. In contrast, inoculated ramets that were totally defoliated did not produce callus but developed blackened necrotic phloem, similar to those depicted in Figure 3D. Mortality among ramets with total defoliation in an actively growing period (June/July) was 90% within 4 wk after inoculation in contrast to 14% among check plants that were 100% defoliated but were not inoculated with fungus (Figure 6). Luttrell (13) reported 80% mortality of 3-moold American elm (Ulmus americana L.) seedlings within 6 mo after inoculation with B. ribis following complete defoliation. The necrotic tissues around the point of inoculations in our study were similar to those described for blackstem diseases caused by C. chrysosperma on cottonwood (P. deltoides Marsh.) (21).

Defoliation in scarlet oaks (Quercus coccinea Muenchh.) caused by insects has been shown to diminish the food reserves needed for tree growth (25). Old et al. (16) reported an extreme susceptibility of two Eucalyptus species to invasion by B. ribis following defoliation. Reduced starch levels in roots and stem tissues due to conversion to glucose and fructose has been reported for defoliated sugar maple trees (26). These sugars may be preferentially channelled to maintaining energy balance for

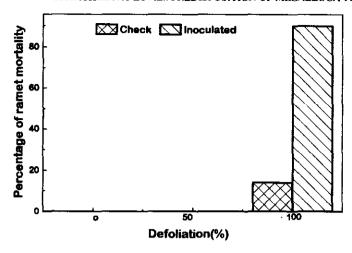


Figure 6. Effects of defoliation on mortality of melaleuca saplings inoculated with B. ribis.

survival, thus reducing their availability for building physical and chemical barriers around wounds. This may explain why defoliated ramets of melaleuca could not resist stem girdling by *B. ribis* when compared to nondefoliated or partially defoliated plants in which callus developed around most wounds.

Our findings on host-pathogen relationship between stressed melaleuca trees and *B. ribis* confirm the reports of other researchers regarding increased canker disease and tree mortality due to 1) an increased moisture stress (3, 7, 27), 2) exposure to low temperature below certain threshold (22, 23, 27), and 3) defoliation (13, 16) on other fungal pathogen and host relationships. We therefore concluded that additional reptitions of experiments were not needed.

We conclude that stress factors such as drought, low temperature, and defoliation, reduce the ability of melaleuca to respond to invasion by *B. ribis*. These results justify further research to test the hypothesis that the ability of this fungus to kill melaleuca trees may be enhanced when inoculation is combined with natural or artificial stresses such as defoliation induced by herbivorous insects or environmentally safe chemicals.

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